MCP-1 Directly Induces Renal Tubulointerstitial Fibrosis Independently of Monocytes/Macrophages Infiltration

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MCP-1 Directly Induces Renal Tubulointerstitial Fibrosis Independently of Monocytes/Macrophages Infiltration

Directed by Professor Shin-Wook Kang

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This certifies that the Doctoral Dissertation of Hye-Young Kang is approved.

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The Graduate School
Yonsei University

December 2014

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Finally and most importantly, I would like to give thanks to God for everything he did for me.

I would like to dedicate this paper to all of you with all of my heart.

Thanks everyone.

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ABSTRACT

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Background: Previous studies have demonstrated the importance of monocyte chemoattractant protein-1 (MCP-1) and its receptor, C-C chemokine receptor 2 (CCR2), in the pathogenesis of various inflammatory and fibrotic diseases via the recruitment and activation of monocytes/macrophages. Recently, however, accumulating in vitro evidence has indicated that MCP-1 per se may act directly on renal cells via CCR2. Therefore, the results of a number of former studies showing the impacts of MCP-1/CCR2 blockade on renal injury may be partly

attributed to a direct inhibitory effect of MCP-1 on renal cells, but it has never been clarified in vivo to date. Since all previous studies to explore the effect of MCP-1 on diverse renal diseases were performed using MCP-1-neutralizing antibody, CCR2 antagonist, or MCP-1 or CCR2 knockout technique, which accompanied a significant reduction in monocytes/macrophages infiltration within the kidney, it was hard to define whether the beneficial influence of MCP-1/CCR2 inhibition on renal injury was attributed to the direct inhibitory effect of MCP-1 on renal cells or was just a consequence of a significant decrease in inflammatory cells infiltration.

Purpose: This study was undertaken to investigate the direct impact of the MCP-1/CCR2 on renal fibrosis in vivo by using monocytes/macrophages-depleted mice. In addition, the direct effect of MCP-1 on extracellular matrix (ECM) synthesis along with the role of CCR2 was also examined in cultured tubular epithelial cells (NRK-52E).

Methods: In vitro, NRK-52E cells were incubated in DMEM media containing 5.6 mM glucose (Control, Con) or recombinant MCP-1 (10 ng/ml) with or without RS102895 (2 and 10 μM), a specific chemical inhibitor of CCR2, and CCR2 siRNA (final concentrations: 10, 25, and 50 nM). After 72 hours, cells were harvested. In vivo, Thirty-six male C57BL/6J mice, weighting 25-30 g, were divided into six groups: Group 1, control mice treated with intravenous

PBS only (N = 6) (Control, Con); Group 2, mice injected with liposome vehicle (LV) and lenti-empty virus intravenously (N = 6); Group 3, mice with liposome-clodronate (LC) and lenti-empty virus (N = 6); Group 4, mice with LV and lenti-MCP-1 virus (N = 6); Group 5, mice with LC and lenti-MCP-1 virus (N = 6); and Group 6, mice with LC, lenti-MCP-1 virus, and RS102895 (N = 6). LV and LC in a volume of 200 µl PBS were injected intravenously every 5 days for 4 weeks, and lenti-empty and lenti-MCP-1 virus were injected intravenously at a dose of 1.5 x 10^7 transfection units every 5 days, three times with LC or LV. RS102895 was delivered at a dose of 3 mg/kg/day for 4 weeks via subcutaneously-implanted osmotic mini-pumps. Either diphtheria toxin (DT) diluted in PBS (10 ng/g of body weight) (N = 24) or PBS (N = 6) was given intraperitoneally to Cd11b-diphtheria toxin receptor (DTR) mice on the day before lenti-MCP-1 virus injection and every 3 days. Lenti-MCP-1 was also injected at 3 days after the first administration. Six from DTR mice injected with lenti-MCP-1 virus and DT were treated with RS102895 (3 mg/kg/day) via osmotic mini-pumps. DTR mice were sacrificed at 10 days after the first lenti-MCP-1 virus injection. The protein expression of fibronectin, type I collagen and CCR2 in cultured NRK-52E cells, and the whole kidney were evaluated by Western blot, and the mRNA expression of fibronectin and type I collagen was assessed by real-time PCR. MCP-1 concentrations in serum and the whole kidney were determined by ELISA. Peripheral blood cell counts were conducted and immunohistochemistry (IHC) for fibronectin, type I collagen and

F4/80 and Masson's trichrome staining were examined.

Results: Compared to Con cells, the protein expression of fibronectin and type I collagen were significantly increased in NRK-52E cells exposed to MCP-1, and these increases were significantly abrogated by RS102895 or CCR2 siRNA ($P < 0.05$). Two days after administration of LC and DT, there was a significant reduction in peripheral blood monocyte counts in C57BL/6J mice and DTR mice, respectively, compared to Con mice ($P < 0.001$). While administration of lenti-MCP-1 virus alone induced a significant increase in the number of infiltrated monocytes/macrophages in the kidney ($P < 0.001$), a concomitant treatment with LC or DT significantly attenuated the increase in monocytes/macrophages infiltration in the kidney ($P < 0.001$) compared to Con mice. The significant increases in fibronectin and type I collagen protein expression ($P < 0.01$), assessed by Western blot, in LC- or DT-treated mice infected with lenti-MCP-1 virus were significantly ameliorated by CCR2 inhibition using osmotic mini-pumps containing RS102895 ($P < 0.05$). The significant increases in IHC staining scores for fibronectin and type I collagen within the tubulointerstitium in monocytes/macrophages-depleted mice infected with lenti-MCP-1 virus ($P < 0.001$) were significantly attenuated by RS102895 treatment ($P < 0.001$).

Conclusions: These findings suggest that the MCP-1/CCR2 system is directly

involved in MCP-1-induced renal fibrosis and blockade of the MCP-1/CCR2 system can be a promising approach to treat various kidney diseases such as diabetic nephropathy, of which MCP-1-induced renal fibrosis is involved in the pathogenesis.

Key words: monocyte chemoattractant protein-1, renal proximal tubular epithelial cells, renal fibrosis, extracellular matrix, monocytes/macrophages

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I. INTRODUCTION

Inflammation is a pathophysiological response to infection or tissue injury[1]. Even though inflammation is fundamentally a beneficial host reaction, uncontrolled or persistent inflammatory process is known to contribute to the pathogenesis of chronic inflammatory diseases, including atherosclerosis[2], systemic sclerosis[3], rheumatoid arthritis[4], and inflammatory bowel disease[5]. Chronic inflammation is also involved in the development and progression of various renal diseases such as diabetic nephropathy[6,7], lupus nephritis[8], and ischemia-reperfusion injury[9]. In all cases, monocytes/macrophages are the

principle cells found at the sites of inflammation.

These cells extravasate from the bloodstream through a process mediated by chemokines secreted from resident cells. Chemokines are a family of chemotactic cytokines that induce the migration of various cell types; to date, more than 40 chemokines have been identified[10]. Among these, monocyte chemoattractant protein (MCP)-1 is the most extensively studied. MCP-1 and its receptor, C-C chemokine receptor 2 (CCR2), have been shown to play an important role in the pathogenesis of various inflammatory and fibrotic diseases via the recruitment and activation of monocytes/macrophages, which are known to release profibrotic cytokines such as transforming growth factor (TGF)-β1, platelet-derived growth factor, and fibroblast growth factor, which in turn modifies the biology of resident cells[11-13]. Based on these facts, numerous investigations were performed and demonstrated that inhibition of the MCP-1/CCR2 system resulted in an improvement of a variety of renal diseases[13-19].

Recently, however, accumulating in vitro evidence has indicated that MCP-1 per se may act directly on various somatic cells, including renal cells, via CCR2[14,20-24]. Previous studies found that MCP-1 induced not only the expression of intercellular adhesion molecule-1, fibronectin, and type IV collagen expression in cultured mesangial cells[20,21,24] but also increased motility and apoptosis in cultured podocytes[23,25,26]. Therefore, the results of a number of former studies showing the impacts of MCP-1/CCR2 blockade on renal injury

may be partly attributed to a direct inhibitory effect of MCP-1 on renal cells, but it has never been clarified in vivo to date. Since all previous studies to explore the effect of MCP-1 on diverse renal diseases were performed using MCP-1-neutralizing antibody[27], CCR2 antagonist[16,28-30], or MCP-1[31] or CCR2 knockout technique[17,28,29], which accompanied a significant reduction in monocytes/macrophages infiltration within the kidney, it was hard to define whether the beneficial influence of MCP-1/CCR2 inhibition on renal injury was attributed to the direct inhibitory effect of MCP-1 on renal cells or was just a consequence of a significant decrease in inflammatory cells infiltration.

In this study, therefore, I tried to clarify the direct impact of the MCP-1/CCR2 system on renal fibrosis in vivo by using monocytes/macrophages-depleted mice. The direct effect of MCP-1 on extracellular matrix (ECM) synthesis along with the role of CCR2 was also examined in cultured tubular epithelial cells.

II. MATERIALS AND METHODS

1. Cell culture

Renal proximal tubular epithelial cells (NRK-52E) were used for cell culture experiments. NRK-52E cells were maintained in Dulbeco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) media supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 26 mM $NaHCO_3$. Cells were grown at 37℃ in humidified 5% CO_2 in air.

2. Inhibition of the MCP-1/CCR2 system

In the current study, two different methods were utilized to block the MCP-1/CCR2 system in these cells; RS102895 (Sigma-Aldrich Corp., St Louis, MO, USA), a specific chemical inhibitor of CCR2, and pre-designed CCR2 siRNA (Ambion, Austin, TX, USA). Purchased CCR2 siRNA was transfected with RNA imax (Invitrogen) according to the manufacturer's protocol. Subconfluent NRK-52E cells were serum restricted for 24 hr, after which the media was replaced by 0.5% FBS DMEM containing 5.6 mM glucose (Control, Con) or recombinant MCP-1 (10 ng/ml) (R&D Systems, Minneapolis, MN, USA) with or without RS102895 (2 and 10 μM) and CCR2 siRNA (final concentrations: 10, 25, and 50 nM). CCR2 siRNA had the following sequences: sense 5'-GGCUAAAAGUAGUGAAUGAtt-3', antisense 5'-UCAUUCACUACUUUUAGCCtt-3'. At 72 hr after media change, cells were harvested. The dose of MCP-1 used in the experiments was determined

based on the results of preliminary experiments.

3. Generation of mouse MCP-1-expressing lentivirus

The lentiviral vector used in the present study was an HIV-based bicistronic vector (GeneCopoeia, Germantown, MD, USA) designated as lenti-MCP-1-GFP, which carried a mouse MCP-1 gene driven by a cytomegalovirus (CMV) immediate-early promoter and an enhanced green fluorescent protein (GFP) reporter gene. Lenti-MCP-1-GFP vectors were prepared by transient transfection of human embryonic kidney (HEK) 293FT cells (Invitrogen) using a lenti-pac HIV expression kit (GeneCopoeia) according to the manufacturer's protocol. Briefly, HEK 293FT cells were cultured in 10 cm tissue culture dishes (BD Biosciences, San Jose, CA, USA). When the cells became confluent, they were transfected with 2.5 μg of lentiviral plasmid along with 2.5 μg of the mixed envelope and packaging plasmids. The viral supernatants were harvested at 48 hr post-transfection, centrifuged at 780 g for 5 min, and filtered through a 0.45 μm pore size filter. After then, additional centrifugation was performed at 83,000 g for 1.5 hr, and the resulting pellet was resuspended in 100 μl of phosphate-buffered saline (PBS). Lentivirus titers were determined by infecting HEK 293FT cells with a dilution series of the viral suspension.

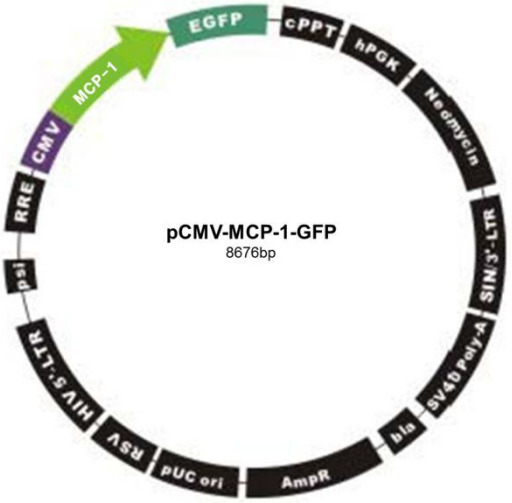


Figure 1. Vector map for the MCP-1-expressing lentiviral vector

4. Animal studies

All animal studies were conducted using a protocol approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University College of Medicine, Seoul, Korea. Liposome-clodronate (LC), which is known to deplete peripheral monocytes/macrophages, was purchased from Sigma-Aldrich Corporation. Thirty-six male C57BL/6J mice, weighting 25-30g, were divided into six groups: Group 1, control mice treated with intravenous PBS only (N = 6) (Control, Con); Group 2, mice injected with liposome vehicle (LV) and lenti-empty virus intravenously (N = 6); Group 3, mice with LC and lenti-empty virus (N = 6); Group 4, mice with LV and lenti-MCP-1 virus (N = 6); Group 5, mice with LC and lenti-MCP-1 virus (N = 6); and Group 6, mice with

LC, lenti-MCP-1 virus, and RS102895 (N = 6). LV and LC in a volume of 200 μl PBS were injected intravenously every 5 days for 4 weeks, and lenti-empty and lenti-MCP-1 virus were injected intravenously at a dose of 1.5 x 10^7 transfection units every 5 days, three times with LC or LV. RS102895 was delivered at a dose of 3 mg/kg/day for 4 weeks via subcutaneously-implanted osmotic mini-pumps (ALZET® Osmotic Pumps; Durect Corp., Cupertino, CA, USA).

Cd11b-diphtheria toxin receptor (DTR) mice on an FVB background [official symbol Tg (ITGAM-DTR/EGFP) 34Lan/J] were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Transient depletion of macrophages was elicited by intraperitoneal administration of DT. The FVB-Tg (ITGAM-DTR/EGFP) 34Lan/J transgenic mice encode a DTR fused with GFP under the control of a human integrin αM (ITGAM) promoter (CD11b). Either DT (Sigma-Aldrich Corp.) diluted in PBS (10 ng/g of body weight) (N = 24) or PBS (N = 6) was given intraperitoneally to mice on the day before lenti-MCP-1 virus injection and every 3 days. Lenti-MCP-1 was also injected at 3 days after the first administration. Six from DTR mice injected with lenti-MCP-1 virus and DT were treated with RS102895 (3 mg/kg/day) via osmotic mini-pumps. DTR mice were sacrificed at 10 days after the first lenti-MCP-1 virus injection.

5. Peripheral blood cell counts

Blood samples were taken three times; at the time of the first LV or DT

injection, 2 days after the first LV or DT injection, and at the time of sacrifice, and monocytes in the peripheral blood were determined.

6. ELISA

Serum and the whole kidney were used for the measurement of MCP-1. The kidney was washed in ice-cold PBS, snap-frozen in liquid nitrogen, pulverized with a mortar and pestle while frozen, and homogenized in 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreithol (DTT), and a broad spectrum proteinase inhibitor cocktail (Boehringer Mannheim GmbH, Mannheim, Germany).

The levels of MCP-1 were determined using commercial ELISA kits (R&D Systems) according to the manufacturer's protocol as previously described. The kit for mouse MCP-1 was species-specific and sensitive up to 5.0 pg/ml.

7. Total RNA extraction and reverse transcription

Total RNA was extracted form cultured NRK-52E cells as previously described, and first strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH). Two μg of total RNA extracted from cultured cells was reverse transcribed using 10 mM random hexanucleotide primer, 1 mM dNTP, 8 mM MgCl2, 30 mM KCl, 50 mM Tris-HCl, pH 8.5, 0.2 mM DTT, 25 U RNAse inhibitor, and 40 U AMV reverse

transcriptase. The mixture was incubated at 30℃ for 10 min and 42℃ for 1 hr, followed by inactivation of enzyme at 99℃ for 5 min.

8. Real-time polymerase chain reaction (Real-time PCR)

The primers used for fibronectin, type I collagen, and 18s amplification were as follows: fibronectin, sense 5'-TGACAACTGCCGTAGACCTG-3', antisense 5'-TACTGGTTGTAGGTGTGGCC-3'; type I collagen, sense 5'-GCATGAGCCGAAGCTAACC-3', antisense 5'-GGAGGTCCACAAAGCTGAAC-3'; and 18s, sense 5'-CCTGCGGCTTAATTTGACTC-3', antisense 5'-AACTAAGAACGGCCATGCAC-3'. Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), PCR was performed with a total volume of 20 μl in each well, containing 10 μl of SYBR Green® PCR Master Mix (Applied Biosystems), 5 μl of cDNA corresponding to 25 ng of RNA, and 5 pmol sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. The PCR conditions were as follows: 35 cycles of denaturation at 94.5℃ for 30 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 1 min. Initial heating at 95℃ for 9 min and final extension at 72℃ for 7 min were performed for all PCR reactions. Each sample was run in triplicate in separate tubes and a control without cDNA was also run in parallel with each assay. After real-time PCR, the temperature was increased from 60 to

95℃ at a rate of 2℃/min to construct a melting curve. The cDNA content of each specimen was determined using a comparative CT method with $2^{-\Delta\Delta CT}$. The results were given as relative expression of fibronectin and type I collagen normalized to the the 18s rRNA and expressed in arbitrary units. Signals from C cells were assigned a relative value of 1.0. In pilot experiments, PCR products revealed a single band on agarose gels.

9. Western blot analysis

The protein expression of fibronectin, type I collagen, and CCR2 in cultured NRK-52E cells, and the whole kidney were evaluated by Western blot as previously described. Cultured cells and the homogenized whole kidney were lysed in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol], treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed in a 10% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), and the membranes were then incubated in blocking buffer A (1 x PBS, 0.1% Tween-20, and 8% nonfat milk) for 1 hr at room temperature, followed by an overnight incubation at 4°C in a 1:1000 dilution of polyclonal antibodies to fibronectin (DAKO, Carpentaria, CA, USA), type I collagen (Southern Biotech, Birmingham, AL, USA), CCR2 (Abcam, Cambridge, UK), or β-actin (Sigma-Aldrich Corp.). The membrane was then

washed once for 15 min and twice for 5 min in 1 x PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, Inc.). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc., Arlington Heights, IL, USA). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany), and the changes in the optical densities of bands from the treated groups relative to Con cells or kidney were used in the analysis.

10. Immunohistochemistry and Masson's trichrome staining

Slices of the kidney were fixed in 10% neutral-buffered formalin, processed in the standard manner, and 5 μm-thick sections of paraffin-embedded tissues were utilized for immunohistochemical (IHC) staining. Slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 min using a Black & Decker vegetable steamer. Primary antibodies for fibronectin and F4/80 (Santa Cruz Biotechnology, Inc.) were diluted to the appropriate concentrations with 2% casein in bovine serum albumin and then added to the slides, followed by an overnight incubation at 4℃. After washing, a secondary antibody was added for 20 min, and the slides were washed and incubated with a tertiary PAP complex for 20 min. Diaminobenzidine was added for 2 min and

the slides were counterstained with hematoxylin. For Masson's trichrome staining, 5 μm-thick sections of paraffin-embedded tissues were deparaffinized, hydrated in ethyl alcohol, washed in tap water, and re-fixed in Bouin's solution at 56℃ for 1 hr. After washing in running tap water for 10 min and staining with Weigert's iron hematoxylin working solution for 10 min, the sections were stained with Biebrich scarlet-acid fuchsin solution for 15 min, followed by a wash for 10 min. Next, the slides were then differentiated in phosphomolybdic-phosphotungstic acid solution for 15 min, transferred to aniline blue solution and stained for 10 min, and after then reacted with 1% acetic acid solution for 5 min. A semi-quantitative score of staining intensity was determined by examining at least twenty tubulointerstitial fields under x 400 magnification. The positive-staining intensity of 1+ to 4+ compared with a negative control (score = 0), in which the IHC staining was performed without the primary antibodies, were defined by one investigator in a blinded fashion, based on the lightness and darkness of the brownish color using a digital image analyzer (MetaMorph version 4.6r5, Universal Imaging, Downingtown, PA). The staining score was obtained by multiplying the intensity of staining by the percentage of tubulointerstitium staining for that intensity; these numbers were then added for each experimental animal to give the staining score [= Σ (intensity of staining) x (% of tubulointerstitium with that intensity)]. The number of F4/80-positive cells was counted in at least 20 fields of the tubulointerstitium/section under x 400 magnification.

11. Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using the Statistical Package for Social Sciences for Windows Ver. 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using one-way ANOVA with *post hoc* test using Bonferonni's method for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

III. RESULTS

1. MCP-1 increases fibronectin expression in NRK-52E cells

First, I examined the impact of MCP-1 on fibronectin expression in cultured NRK-52E cells. The administration of recombinant MCP-1 significantly increased fibronectin protein expression in NRK-52E cells in a dose-dependent manner ($P < 0.05$) (Fig. 2).

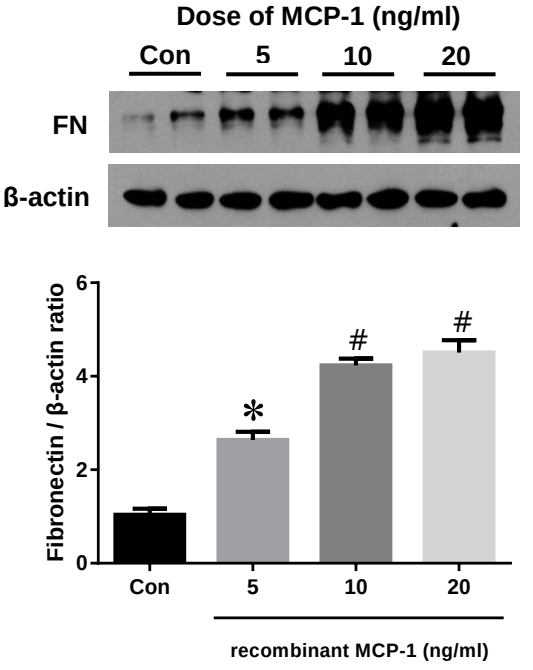


Figure 2. A representative Western blot for fibronectin protein in NRK-52E cells exposed to 5 ng/ml, 10 ng/ml, or 20 ng/ml MCP-1 (representative of five blots). MCP-1 significantly increased fibronectin protein expression in NRK-52E cells in a dependent manner.

*; $P < 0.05$ vs. Con, #; $P < 0.01$ vs. Con

2. CCR2 inhibition ameliorates MCP-1-induced fibronectin and type I collagen expression

Second, to clarify the role of CCR2 in MCP-1-induced fibronectin expression, the cells were treated with RS102895, a chemical inhibitor of CCR2, or CCR2 siRNA. The efficacy of CCR2 siRNA transfection was evaluated by determining the protein expression of CCR2. As shown in Fig. 3, CCR2 protein expression was significantly decreased in CCR2 siRNA-transfected NRK-52E cells in a dose-dependent manner ($P < 0.05$). In contrast, a negative control of the CCR2 siRNA had no effect on the expression of CCR2 protein.

The administration of 10 ng/ml recombinant MCP-1 significantly increased the expression of fibronectin and type I collagen protein in cultured NRK-52E cells. The protein expression of fibronectin and type I collagen were 4.1- and 2.8-fold higher in MCP-1-treated NRK-52E cells, respectively, compared to Con cells ($P < 0.01$), and these increases in fibronectin and type I collagen protein expression were significantly attenuated by CCR2 inhibition with 10 μM RS102895 or 25 nM CCR2 siRNA ($P < 0.01$) (Fig. 4A), suggesting that MCP-1 directly induced fibronectin and type I collagen protein expression in NRK-52E cells via CCR2. Meanwhile, MCP-1 had no effect on the expression of CCR2 protein in these cells.

Real-time PCR also revealed that MCP-1 significantly increased fibronectin and type I collagen mRNA expression ($P < 0.01$), which were significantly abrogated by 10 μM RS102895 or 25 nM CCR2 siRNA ($P < 0.01$) (Fig. 4B).

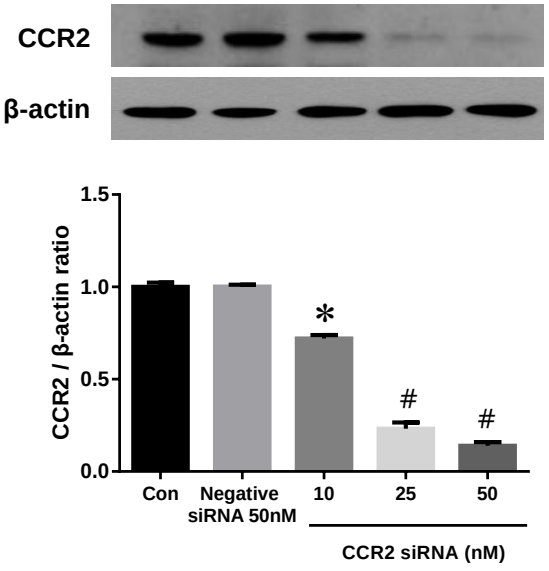


Figure 3. A representative Western blot for CCR2 protein in NRK-52E cells transfected with 10, 25, or 50 nM CCR2 siRNA (representative of five blots). CCR2 protein expression was significantly decreased in CCR2 siRNA-transfected NRK-52E cells in a dose-dependent manner. In contrast, a negative control of CCR2 siRNA (50 nM) had no effect on CCR2 protein expression.

*; $P < 0.05$ vs. Con, #; $P < 0.001$ vs. Con

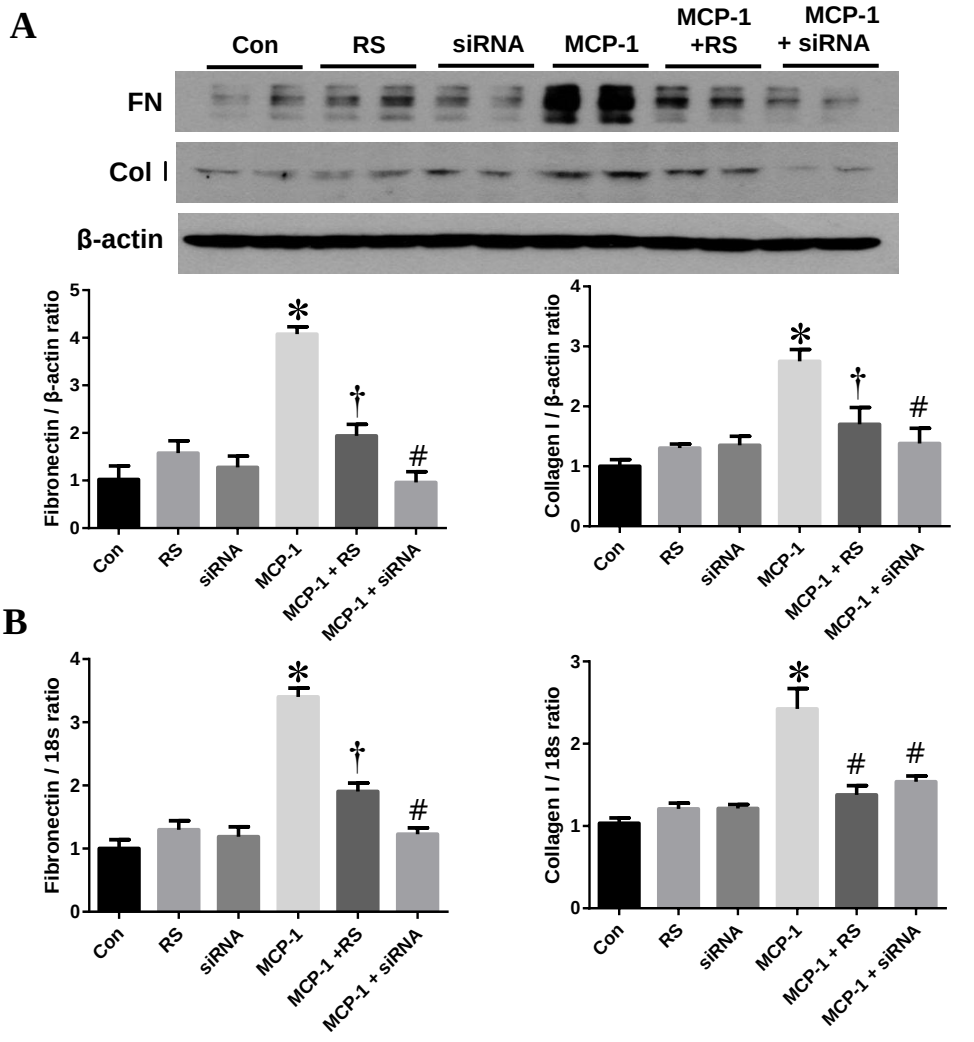
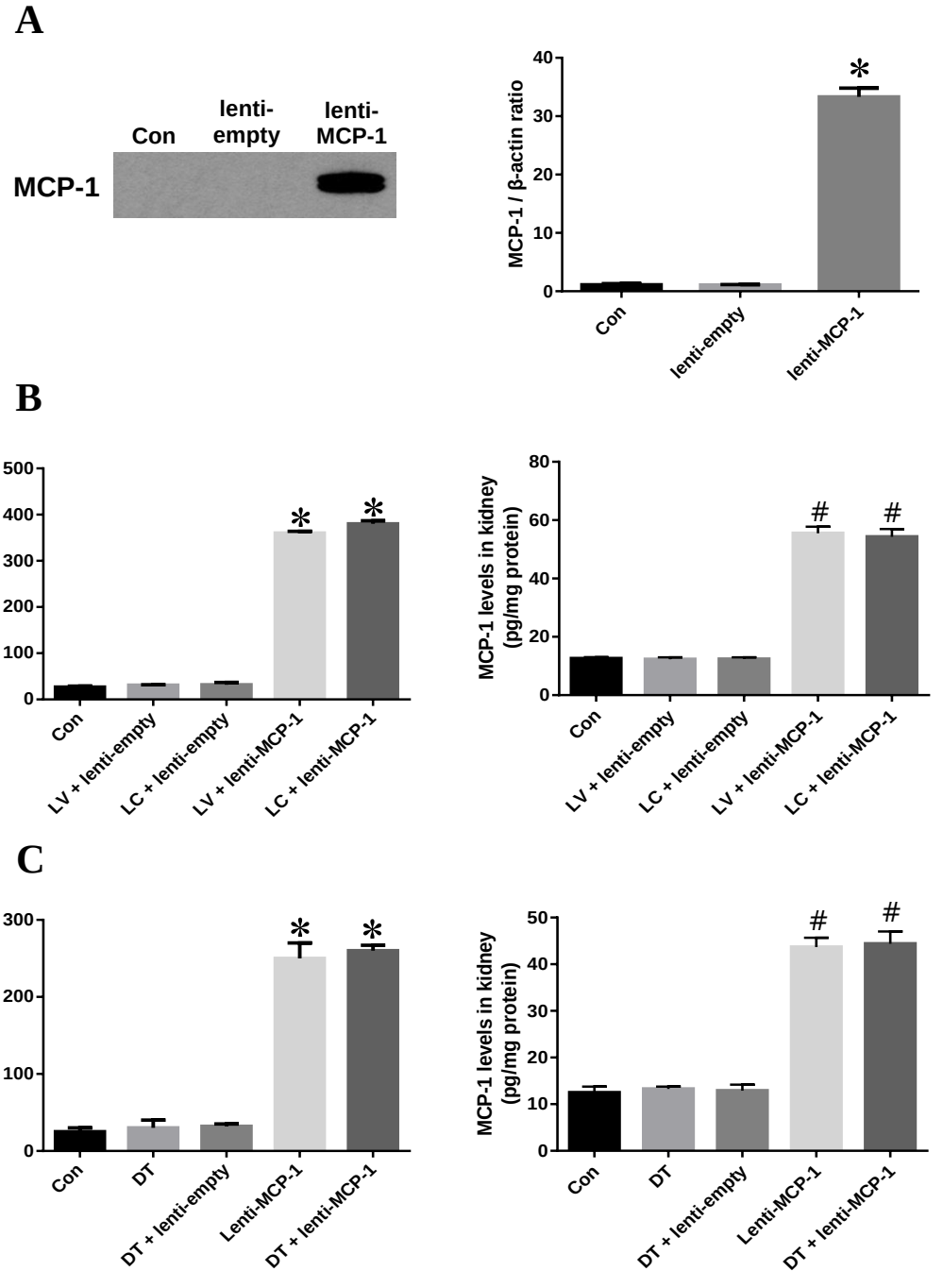


Figure 4. Fibronectin and type I collagen expression in NRK-52E cells exposed to 10 μM RS102895 (RS), 25 nM CCR2 siRNA (siRNA), 10 ng/ml MCP-1 (MCP-1), MCP-1+RS, or MCP-1+siRNA. (A) A representative Western blot for fibronectin and type I collagen (representative of five blots). The protein expression of fibronectin and type I collagen were significantly increased in NRK-52E cells exposed to MCP-1, and these increases were significantly abrogated by RS or siRNA. (B) Real-time PCR also revealed that MCP-1 significantly increased fibronectin and type I collagen mRNA expression, which were significantly abrogated by 10 μM RS102895 or 25 nM CCR2 siRNA.

*; $P < 0.01$ vs. Con, †; $P < 0.05$ vs. MCP-1, #; $P < 0.01$ vs. MCP-1

3. MCP-1-expressing lentivirus increases MCP-1 protein both in vitro and in vivo

Next, to test the efficacy of MCP-1-expressing lentivirus, the levels of MCP-1 were assessed in NRK-52E cells infected with lenti-MCP-1 virus, and in serum and the whole kidney collected from mice injected with lenti-MCP-1 virus by Western blot and ELISA, respectively. MCP-1 protein expression was significantly increased in lenti-MCP-1-infected NRK-52E cells compared to Con cells ($P < 0.001$) (Fig. 5A). MCP-1 concentrations in serum and the whole kidney were also significantly higher in LC or DT-treated mice, in which peripheral monocytes/macrophages were depleted, injected with lenti-MCP-1 virus compared to Con mice ($P < 0.01$) (Fig. 5B, C).


A
Con
lenti-empty
lenti-MCP-1
MCP-1
MCP-1 / β-actin ratio
40
30
20
10
0
*
Con
lenti-empty
lenti-MCP-1
B
MCP-1 levels (pg/ml)
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400
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200
100
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Con
LV + lenti-empty
LC + lenti-empty
LV + lenti-MCP-1
LC + lenti-MCP-1
MCP-1 levels in kidney (pg/mg protein)
80
60
40
20
0
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Con
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LC + lenti-empty
LV + lenti-MCP-1
LC + lenti-MCP-1
C
MCP-1 levels (pg/ml)
300
200
100
0
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DT
DT + lenti-empty
Lenti-MCP-1
DT + lenti-MCP-1
MCP-1 levels in kidney (pg/mg protein)
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0
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DT + lenti-empty
Lenti-MCP-1
DT + lenti-MCP-1

Figure 5. The levels of MCP-1 in NRK-52E cells infected with lenti-MCP-1 virus, and in serum and the whole kidney collected from mice injected with lenti-MCP-1 virus. (A) A representative Western blot for MCP-1 in NRK-52E cells infected with lenti-empty or lenti-MCP-1 virus (representative of five blots). The protein expression of MCP-1 was significantly increased in lenti-MCP-1-infected NRK-52E cells, while lenti-empty virus had no effect on MCP-1 protein expression. (B) MCP-1 levels, assessed by ELISA, in mice treated with PBS (Con), liposome vehicle (LV)+lenti-empty, liposome-clodronate (LC)+lenti-empty, LV+lenti-MCP-1, or LC+lenti-MCP-1. There were significant increases in MCP-1 concentrations in serum and the whole kidney of mice treated with LV+lenti-MCP-1 compared to Con, but these increases were not affected by LC. (C) MCP-1 levels, assessed by ELISA, in Cd11b-diphtheria toxin receptor (DTR) mice treated with PBS (Con), DT, DT+lenti-empty, lenti-MCP-1, or DT+lenti-MCP-1. There were significant increases in MCP-1 levels in serum and the whole kidney of mice treated with DT+lenti-MCP-1 compared to Con, but DT had no impact on these increases.

*; $P < 0.001$ vs. Con, #; $P < 0.01$ vs. Con

4. LC and DT nearly deplete peripheral blood monocytes, and macrophages infiltration in the kidney

Two days after intravenous administration of LC, there was a significant reduction in peripheral blood monocyte counts ($P < 0.001$) (Fig. 6A). The proportions of monocytes were 5.6 ± 0.8% and 6.2 ± 0.3% in PBS- and LV-treated mice, respectively, while it was significantly decreased to 1.0 ± 0.2% after LC treatment. Similar finding was observed in DT-injected mice ($P < 0.001$) (Fig. 6B).

Ablation of monocyte/macrophage was also confirmed in the kidney by IHC staining for F4/80. While administration of lenti-MCP-1 virus alone induced a significant increase in the number of infiltrated monocytes/macrophages in the kidney ($P < 0.001$), a concomitant treatment with LC or DT significantly attenuated the increase in monocytes/macrophages infiltration in the kidney ($P < 0.001$) (Fig. 7).

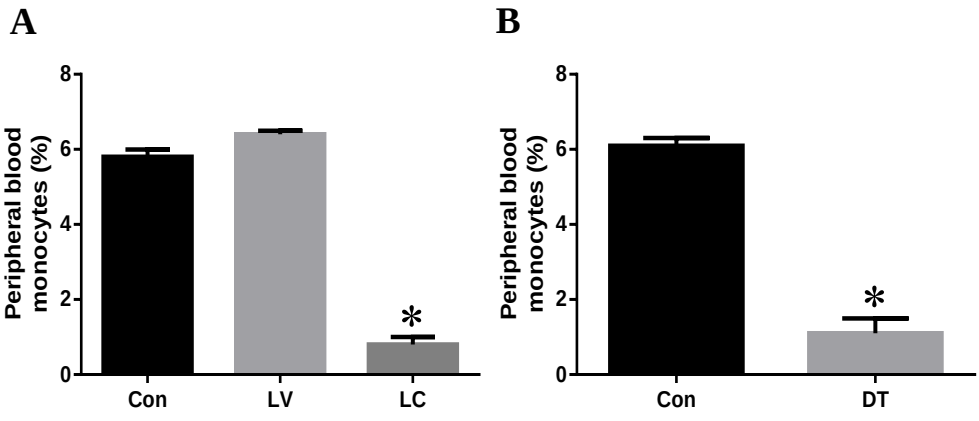
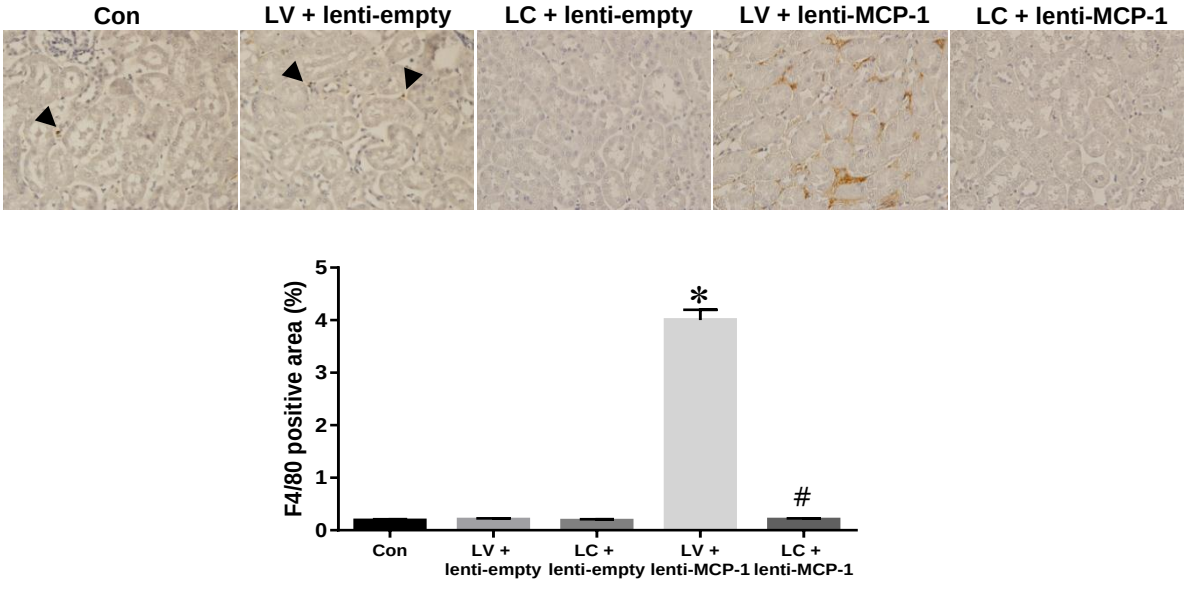


Figure 6. Monocyte counts in the peripheral blood. (A) Monocyte counts in mice treated with PBS (Con), liposome vehicle (LV), or liposome-clodronate (LC). Two days after intravenous administration of LC, there was a significant reduction in peripheral blood monocyte counts. (B) Monocyte counts in in Cd11b-diphtheria toxin receptor (DTR) mice treated with PBS (Con) or DT. Compared to Con, peripheral blood monocyte counts were significantly decreased in DT-treated mice.

*; $P < 0.001$ vs. Con

A


Con
LV + lenti-empty
LC + lenti-empty
LV + lenti-MCP-1
LC + lenti-MCP-1
F4/80 positive area (%)
0
1
2
3
4
5
*
#
Con
LV + lenti-empty
LC + lenti-empty
LV + lenti-MCP-1
LC + lenti-MCP-1

B

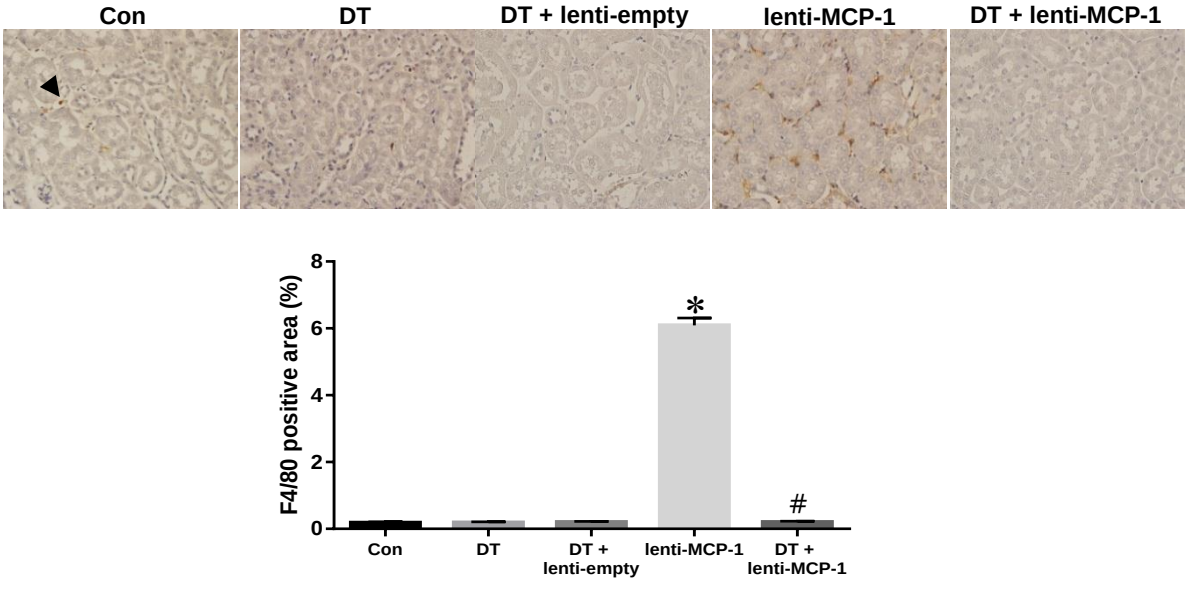
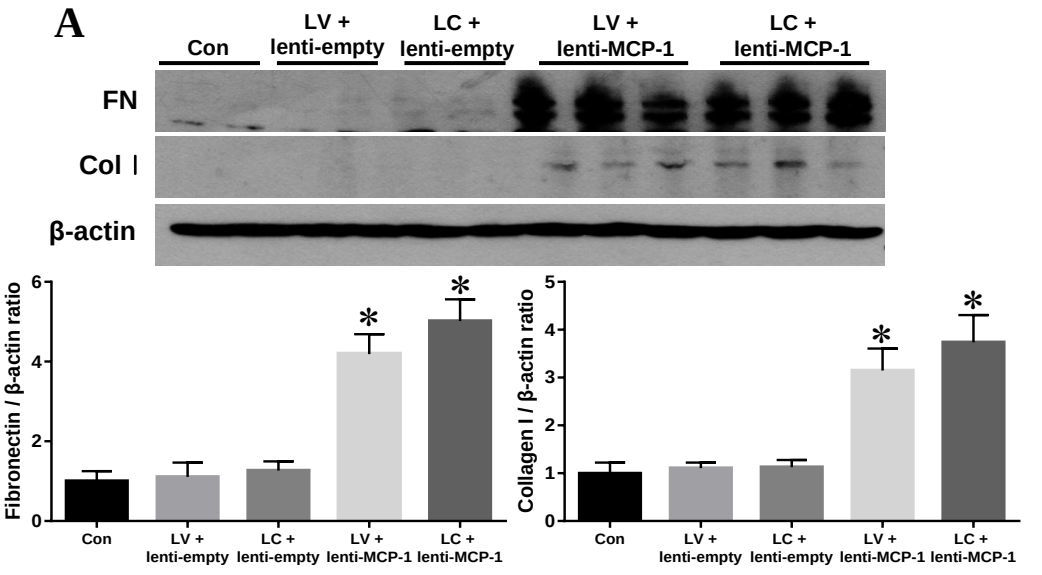

Con
DT
DT + lenti-empty
lenti-MCP-1
DT + lenti-MCP-1
F4/80 positive area (%)
0
2
4
6
8
*
#
Con
DT
DT + lenti-empty
lenti-MCP-1
DT + lenti-MCP-1

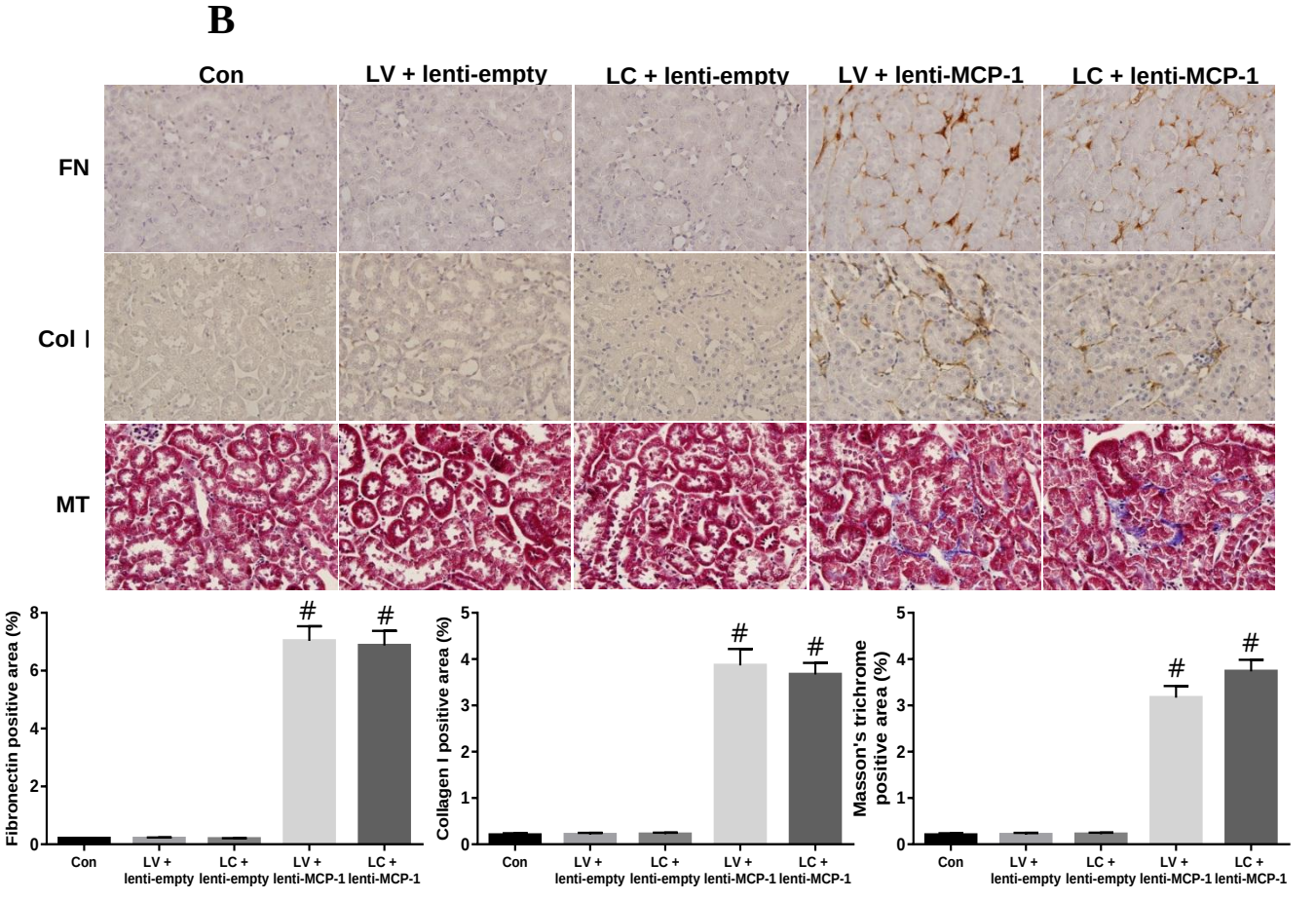
Figure 7. Immunohistochemical staining for F4/80 with the kidney tissue. (A) A representative photograph of the renal tubulointerstitium in mice treated with PBS (Con), liposome vehicle (LV)+lenti-empty, liposome-clodronate (LC)+lenti-empty, LV+lenti-MCP-1, or LC+lenti-MCP-1. Lenti-MCP-1 infection significantly increased macrophage infiltration within the kidney, which was significantly ameliorated by LC. (B) A representative photograph of the renal tubulointerstitium in Cd11b-diphtheria toxin receptor (DTR) mice treated with Con, DT, DT+lenti-empty, lenti-MCP-1, or DT+lenti-MCP-1. Macrophage infiltration within the kidney induced by lenti-MCP-1 was significantly attenuated in mice treated with DT (x 400).

*; $P < 0.001$ vs. Con, #; $P < 0.001$ vs. LV+lenti-MCP-1 or lenti-MCP-1

5. Lenti-MCP-1 virus induces ECM accumulation in the kidney independently of monocytes/macrophages infiltration

Compared to Con mice, fibronectin and type I collagen protein expression in the kidney, assessed by Western blot and IHC staining, were significantly increased in mice infected by not only lenti-MCP-1 virus but also lenti-MCP-1 virus with LC ($P < 0.01$) (Fig. 8A, B). The protein expression of fibronectin and type I collagen was also significantly increased in mice infected by lenti-MCP-1 virus along with saline or DT compared to Con mice, even though monocytes/macrophages were nearly ablated by DT ($P < 0.01$) (Fig. 8C, D). In contrast, lenti-empty virus had no effect on fibronectin or type I collagen protein expression in mice. Masson's trichome staining showed a similar pattern (Fig. 8B, D).


A
Con
LV + lenti-empty
LC + lenti-empty
LV + lenti-MCP-1
LC + lenti-MCP-1
FN
Col Ⅰ
β-actin
Fibronectin / β-actin ratio
Collagen Ⅰ / β-actin ratio
*


B
Con
LV + lenti-empty
LC + lenti-empty
LV + lenti-MCP-1
LC + lenti-MCP-1
FN
Col Ⅰ
MT
Fibronectin positive area (%)
Collagen I positive area (%)
Masson's trichrome positive area (%)
#

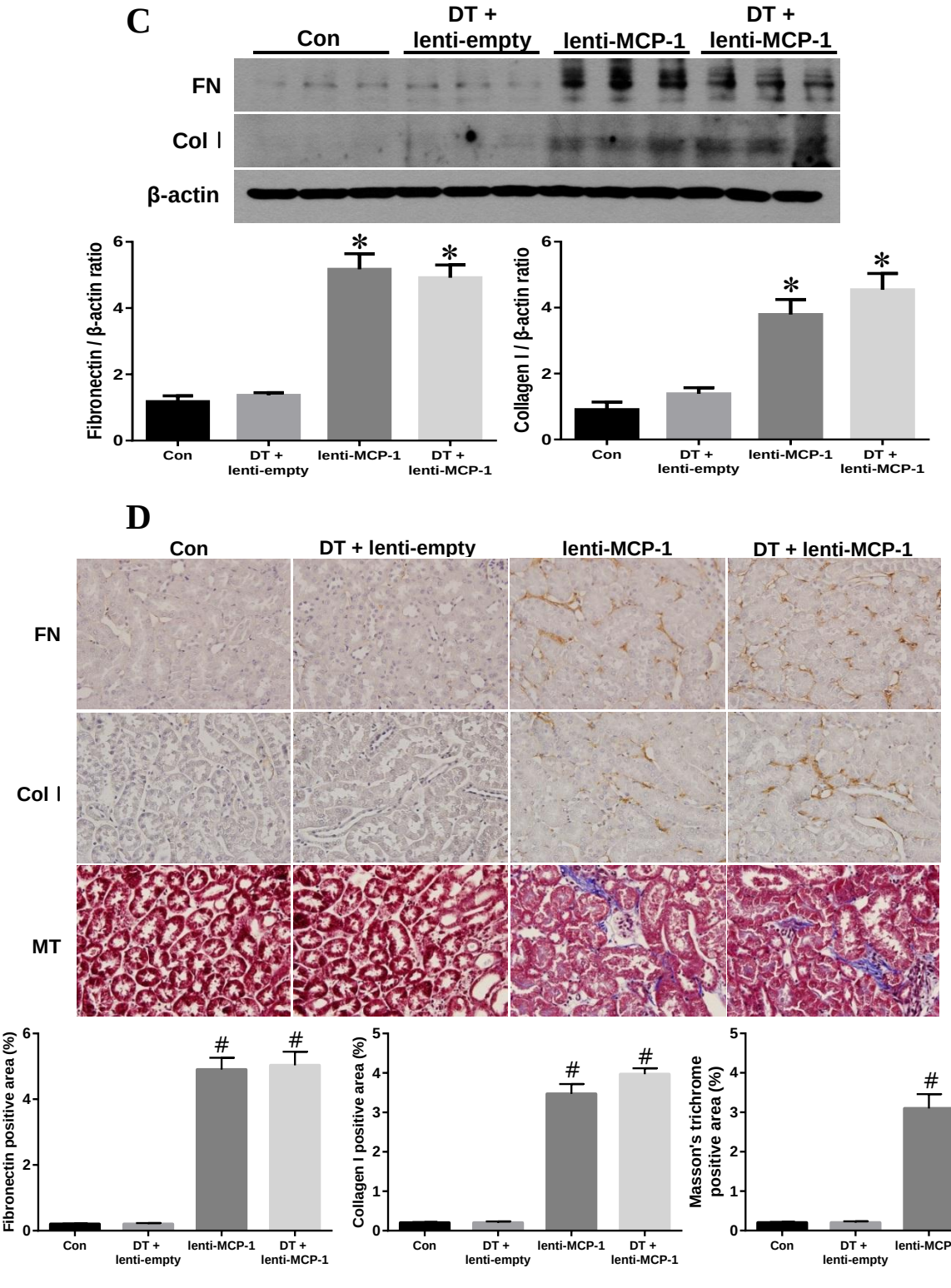
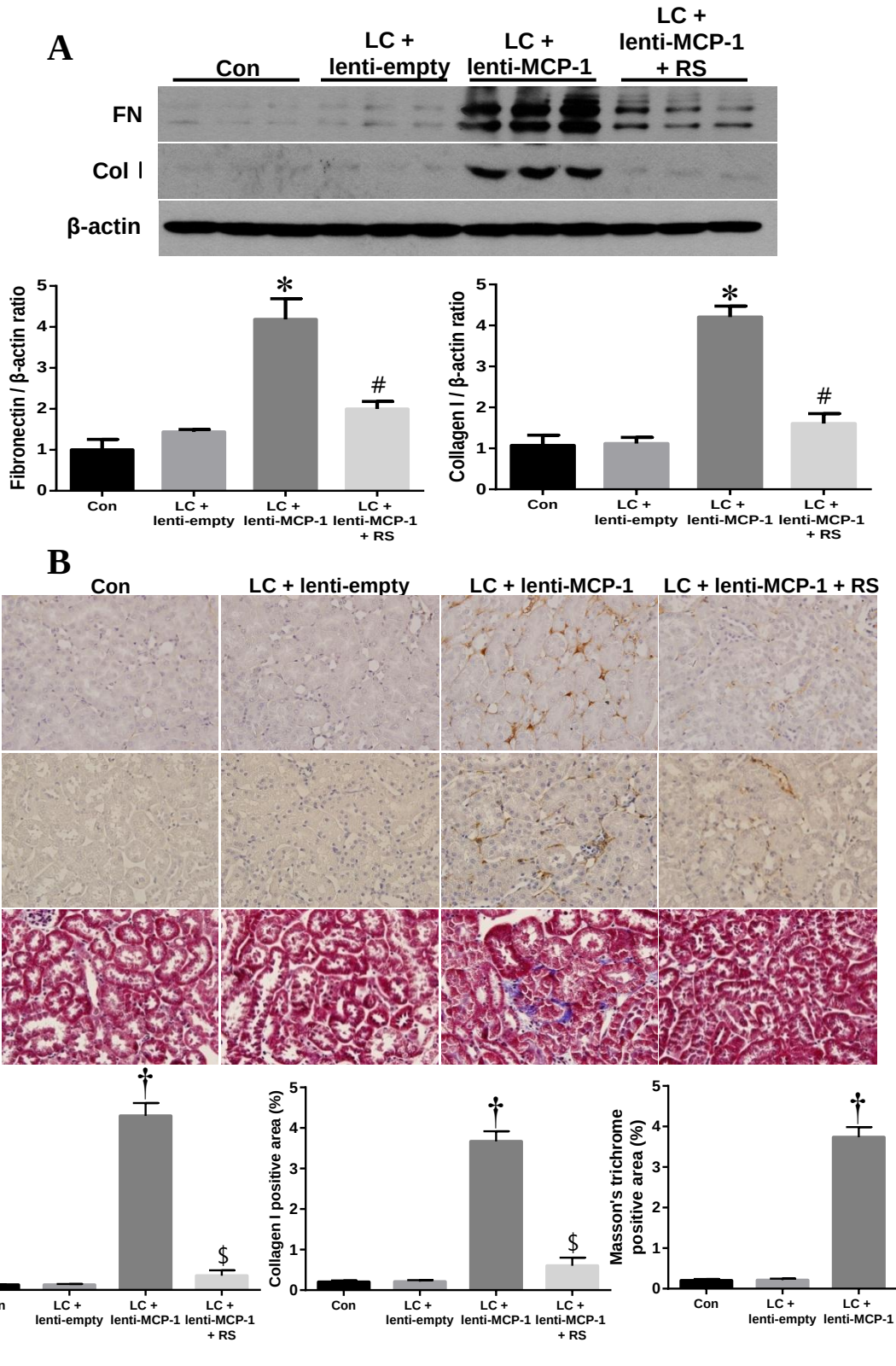

C
Con
DT + lenti-empty
lenti-MCP-1
DT + lenti-MCP-1
FN
Col Ⅰ
β-actin
Fibronectin / β-actin ratio
Collagen Ⅰ / β-actin ratio
Con
DT + lenti-empty
lenti-MCP-1
DT + lenti-MCP-1
D
Con
DT + lenti-empty
lenti-MCP-1
DT + lenti-MCP-1
FN
Col Ⅰ
MT
Fibronectin positive area (%)
Collagen I positive area (%)
Masson's trichrome positive area (%)

Figure 8. ECM protein expression by lenti-MCP-1 virus in the kidney of monocytes/macrophages-depleted mice. (A) A representative Western blot for fibronectin and type I collagen in mice treated with PBS (Con), liposome vehicle (LV)+lenti-empty, liposome-clodronate (LC)+lenti-empty, LV+lenti-MCP-1, or LC+lenti-MCP-1 (representative of five blots). Fibronectin and type I collagen protein expression were significantly increased in not only LV+lenti-MCP-1 but also LC+lenti-MCP-1 mice compared to Con mice. (B) Immunohistochemical staining for fibronectin and type I collagen, and Masson's trichrome staining also revealed that the protein expression of fibronectin and type I collagen, and fibrosis, respectively, were significantly increased in mice infected with lenti-MCP-1. Even though LC nearly depleted macrophages infiltration within the kidney, it had no effect on fibronectin and type I collagen expression, or fibrosis in lenti-MCP-1-injected mice (x 400). (C) A representative Western blot for fibronectin and type I collagen in Cd11b-diphtheria toxin receptor (DTR) mice treated with Con, DT+lenti-empty, lenti-MCP-1, or DT+lenti-MCP-1 (representative of five blots). The expression of fibronectin and type I collagen protein were significantly increased in lenti-MCP-1 as well as DT+lenti-MCP-1 mice compared to Con mice. (D) Immunohistochemical staining for fibronectin and type I collagen, and Masson's trichrome staining also demonstrated that fibronectin and type I collagen protein expression, and fibrosis, respectively, were significantly increased in mice injected with lenti-MCP-1 or DT+lenti-MCP-1, although monocytes/macrophages were nearly ablated by DT (x 400).

*; $P < 0.01$ vs. Con, #; $P < 0.001$ vs. Con

6. CCR2 inhibitor abrogates MCP-1-induced ECM accumulation in the kidney

The significant increases in fibronectin and type I collagen protein expression ($P < 0.01$), assessed by Western blot, in LC- or DT-treated mice infected with lenti-MCP-1 virus were significantly ameliorated by CCR2 inhibition using osmotic mini-pumps containing RS102895 ($P < 0.05$) (Fig 9A, 9C). IHC staining for fibronectin and type I collagen protein also confirmed the Western blot findings. The significant increases in IHC staining scores for fibronectin and type I collagen within the tubulointerstitium in monocytes/macrophages-depleted mice infected with lenti-MCP-1 virus ($P < 0.001$) were significantly attenuated by RS102895 treatment ($P < 0.001$) (Fig. 9B, 9D).


A
Con
LC + lenti-empty
LC + lenti-MCP-1
LC + lenti-MCP-1 + RS
FN
Col Ⅰ
β-actin
Fibronectin / β-actin ratio
Collagen Ⅰ / β-actin ratio
B
Con
LC + lenti-empty
LC + lenti-MCP-1
LC + lenti-MCP-1 + RS
FN
Col Ⅰ
MT
Fibronectin positive area (%)
Collagen I positive area (%)
Masson's trichrome positive area (%)

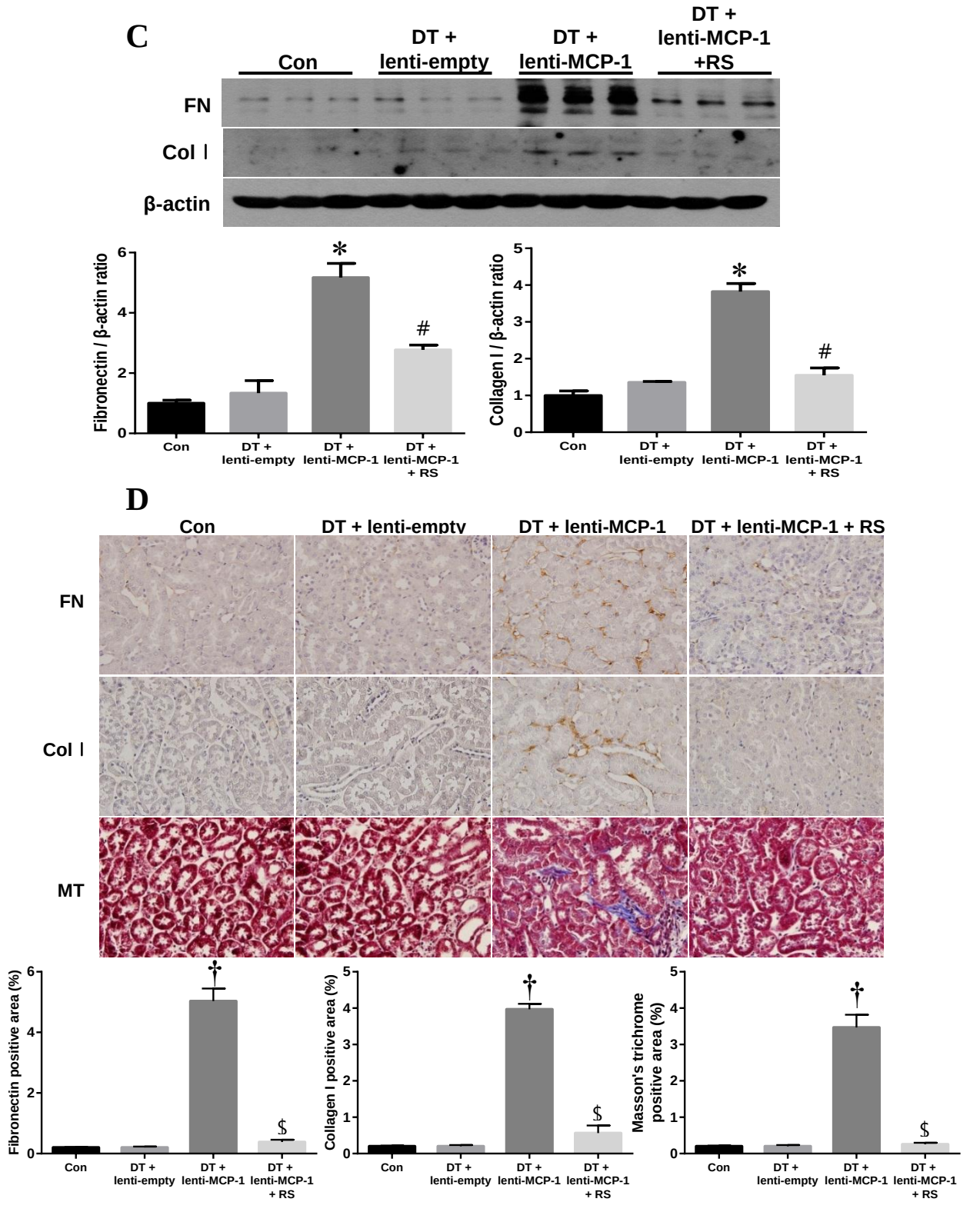

C
Con
DT + lenti-empty
DT + lenti-MCP-1
DT + lenti-MCP-1 +RS
FN
Col Ⅰ
β-actin
Fibronectin / β-actin ratio
Collagen Ⅰ / β-actin ratio
Con
DT + lenti-empty
DT + lenti-MCP-1
DT + lenti-MCP-1 + RS
D
Con
DT + lenti-empty
DT + lenti-MCP-1
DT + lenti-MCP-1 + RS
FN
Col Ⅰ
MT
Fibronectin positive area (%)
Collagen I positive area (%)
Masson's trichrome positive area (%)

Figure 9. The effect of CCR2 inhibition on lenti-MCP-1 virus-induced ECM protein expression in the kidney regardless of monocytes/macrophages infiltration. (A) A representative Western blot for fibronectin and type I collagen in mice treated with PBS (Con), liposome-clodronate (LC)+lenti-empty, LC+lenti-MCP-1, or LC+lenti-MCP-1+RS102895 (3 mg/kg/day) (RS) (representative of five blots). The significant increases in the protein expression of fibronectin and type I collagen induced by MCP-1 were significantly ameliorated in LC+lenti-MCP-1 mice treated by RS. (B) Immunohistochemical staining for fibronectin and type I collagen, and Masson's trichrome staining also found that MCP-1-induced fibronectin and type I collagen protein expression, and fibrosis, respectively, were significantly attenuated by RS (x 400). (C) A representative Western blot for fibronectin and type I collagen in Cd11b-diphtheria toxin receptor (DTR) mice treated with Con, DT+lenti-empty, DT+lenti-MCP-1, or DT+lenti-MCP-1+RS (representative of five blots). The significant increases in the expression of fibronectin and type I collagen protein in DT+lenti-MCP-1-injected mice were significantly inhibited by RS treatment. (D) Immunohistochemical staining for fibronectin and type I collagen, and Masson's trichrome staining also showed that the increases in the protein expression of fibronectin and type I collagen, and fibrosis, respectively, in DT+lenti-MCP-1 mice were significantly abrogated by the administration of RS (x 400).

*; $P < 0.01$ vs. Con, #; $P < 0.05$ vs. LC+lenti-MCP-1 or DT+lenti-MCP-1, †; $P < 0.001$ vs. Con, $; $P < 0.001$ vs. LC+lenti-MCP-1

IV. DISCUSSION

MCP-1, a well-known chemokine, and its receptor CCR2 have been found to be involved in the pathogenesis of various kidney diseases via the recruitment of monocytes/macrophages[13-16,19]. Recently, however, accumulating in vitro evidence indicates that the MCP-1/CCR2 system per se has a direct effect on renal cells[20-24]. In this study, I demonstrate for the first time that MCP-1 increases ECM synthesis in cultured renal tubular epithelial cells. In addition, the results of my in vivo experiments show that enhanced MCP-1 expression in the kidney is associated with ECM accumulation even in the absence of monocytes/macrophages infiltration.

Previous clinical and experimental studies have suggested that the MCP-1/CCR2 system plays a major role in the development of numerous diseases in the whole body, including inflammatory bowel disease[5], atherosclerosis[2], asthma[32], ischemia-related neuronal death[33], systemic sclerosis[3], and rheumatoid arthritis[4]. Regarding the kidney diseases, specifically, it is known to be involved in the pathogenesis of diabetic nephropathy[16,19], lupus nephritis[8], ischemia-reperfusion injury[9], and crescentic glomerulonephritis[14]. The levels of urinary MCP-1, which represents intrarenal MCP-1 production, were significantly increased in diabetic patients with overt nephropathy and advanced tubulointerstitial lesions[34,35]. There was also a significant association of urinary MCP-1 levels with the percentage of crescents and the number of CD68-positive infiltrating cells in the interstitium in patients with crescentic

glomerulonephritis[36]. Furthermore, urinary MCP-1 was revealed to be an independent marker of renal function decline in diabetic and non-diabetic proteinuric diseases[37]. Since an increase in MCP-1 expression is demonstrated mainly in the tubulointerstitial area in diverse renal disease, including diabetic nephropathy[35,38], lupus nephritis[39,40], and IgA nephropathy[41], and the severity of tubulointerstitial lesions correlates with the renal function better than the extent of glomerular injury[42], it is inferred that MCP-1 contributes to the development of tubulointerstitial injury in a variety of renal diseases. The results of the current study showed that MCP-1-expressing lentivirus increased fibronectin and type I collagen expression in cultured tubular epithelial cells. Intravenous injection of MCP-1-expressing lentivirus to mice also successfully induced MCP-1 expression in the kidney, which was accompanied with ECM accumulation and monocytes/macrophages infiltration. In addition, these changes in ECM protein expression were abrogated by a CCR2 inhibitor. Taken together, I assumed that overexpression of MCP-1 in tubular epithelial cells both in vitro and in vivo led to an increase in ECM synthesis via CCR2.

MCP-1 recruits and activates monocytes/macrophages. These infiltrated inflammatory cells are known to release profibrotic cytokines such as transforming growth factor (TGF)-β1, platelet-derived growth factor, and fibroblast growth factor[43-45], which in turn stimulates the resident cells to synthesize ECM. In the kidney, like other organs, enhanced MCP-1 expression was associated with an increase in inflammatory cells infiltration and ECM

accumulation[11-13]. Based on these findings, innumerous investigations have been performed to explore the effect of MCP-1/CCR2 inhibition on renal diseases. Treatment with a neutralizing antibody to MCP-1 significantly reduced proteinuria and interstitial fibrosis in an animal model of nephrotoxic nephritis[46-48]. Tubulointerstitial injury associated with nephrotoxic serum nephritis was also significantly ameliorated in MCP-1-deficient mice[49]. Moreover, either N-terminal deletion mutant of the human MCP-1 gene (7ND); which was constructed to competitively inhibit native MCP-1 action[50], or a CCR2 inhibitor significantly attenuated ECM accumulation in animals with renal fibrosis induced by unilateral ureteral obstruction (UUO)[13]. Furthermore, renal interstitial fibrosis along with albuminuria was significantly abrogated in MCP-1-deficient animal models of type 1[19,21] and type 2 diabetes[51]. Similarly, CCR2 knockout mice were significantly protected from renal injury induced by diabetes[52,53], systemic lupus erythematosus[17], ischemia-reperfusion[29], or UUO[28]. All these beneficial effects of inhibiting the MCP-1/CCR2 system were accompanied by a significant reduction in monocytes/macrophages infiltration within the kidney, suggesting monocytes/macrophages were mainly responsible for these renal injuries.

Recently, however, mounting in vitro evidence shows that MCP-1 exerts direct effects on renal cells via CCR2[14,21-24]. MCP-1 increased intercellular adhesion molecule-1 (ICAM-1) and fibronectin expression in cultured mesangial cells[20]. In addition, the MCP-1/CCR2 system was found to be

involved in apoptosis[23], cellular motility[25,26], rearrangement of the actin cytoskeleton[54], and albumin permeability[26] in cultured podocytes. Nephrin mRNA and protein expression was also significantly decreased in MCP-1-treated podocytes[31]. In cultured tubular epithelial cells, moreover, MCP-1 binding to CCR2 stimulated interleukin-6 secretion and ICAM-1 synthesis in a time- and dose-dependent manner[55], and induced epithelial-mesenchymal transition through the ERK pathway[56]. The present study demonstrated for the first time that MCP-1 directly induced fibronectin mRNA and protein expression in cultured tubular epithelial cells, and this increase in fibronectin expression was significantly ameliorated by a CCR2 inhibitor. Considering the results of the previous and present studies, it was surmised that the beneficial in vivo effect of MCP-1/CCR2 inhibition on experimental renal diseases could be attributed to the alleviation of the deleterious impact of MCP-1 on renal cells rather than reduced inflammatory cells infiltration.

To verify this hypothesis, two different methods were employed to exclude the influence of monocytes/macrophages in the kidney overexpressed with MCP-1. First, DTR transgenic mice were used. Since Duffield et al developed this transgenic mouse[57], it has been widely used to investigate the role of monocytes/macrophages in renal injury induced by diabetic nephropathy[58], UUO[59], and nephrotoxic serum[60]. DT injection to DTR transgenic mice significantly reduced the number of monocytes/macrophages in the peripheral

blood and kidney, but the concomitant administration of DT and MCP-1-expressing lentivirus significantly increased the mortality after 15 days. Therefore, the experiments with DTR transgenic mice were completed at 10 days. Second, LC was used. Even though neutrophil depletion and ineffective depletion of monocytes/macrophages in some tissues have been reported with LC[61,62], this procedure has also been widely applied to explore the influence of monocytes/macrophages in various experimental renal diseases, including acute glomerulonephritis[63], acute kidney injury associated with ischemia-reperfusion[64,65], and renal interstitial fibrosis induced by UUO[66,67]. In this study, monocytes/macrophages were nearly completely depleted in the peripheral blood and kidney by the administration of LC, which was in accordance with the results of previous studies[63-67]. Furthermore, I observed that renal ECM protein expression was significantly increased in mice injected with MCP-1-expressing lentivirus, and this increase still remained significant even when monocytes/macrophages were depleted. These findings suggest that MCP-1 per se induces renal tubulointerstitial fibrosis in vivo, regardless of inflammatory cells infiltration.

In summary, mRNA and protein expression of fibronectin and type I collagen were significantly increased by MCP-1 in cultured NRK-52E cells via CCR2. In addition, MCP-1 induced renal tubulointerstitial fibrosis, independently of monocytes/macrophages infiltration within the kidney. These findings suggest that blockade of the MCP-1/CCR2 system can be a promising approach to treat

various kidney diseases such as diabetic nephropathy, of which MCP-1-induced renal fibrosis is involved in the pathogenesis.

V. CONCLUSION

MCP-1 significantly increased mRNA and protein expression of fibronectin and type I collagen in cultured renal tubular epithelial cells and in mouse kidney via CCR2 independently of monocytes/macrophages infiltration. These findings suggest that blockade of the MCP-1/CCR2 system can be a promising approach to treat various kidney diseases such as diabetic nephropathy, of which MCP-1-induced renal fibrosis is involved in the pathogenesis.

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ABSTRACT (IN KOREAN)

MCP-1/CCR2 시스템이 신섬유화에 미치는 직접적 영향

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배경: 조직 내 단핵구/대식세포의 침윤은 염증반응에서 흔히 관찰되는 소견으로, 이러한 세포들의 침윤을 통하여 염증반응 및 섬유화가 동반되는 각종 질환이 발생하거나 진행되는 것으로 알려져 있다. 단핵구/대식세포의 침윤은 주화성 물질의 유도에 의하여 일어나게 되는데, 이러한 물질 중에서 가장 대표적인 것이 monocyte chemoattractant protein-1 (MCP-1)이다. 최근의 연구들에 의하면 일부 세포에서 MCP-1의 수용체인 CCR2의 존재가 확인되었으며, MCP-1이 염증세포의 침윤 유도 이외에도 이들 세포에 직접적으로 작용하는 것으로 알려져 있다. 그러나, 현재까지 MCP-1의 병인적 역할을

규명한 모든 국내외 연구들을 보면, MCP-1 또는 CCR2 녹아웃 마우스, MCP-1 중화 항체, 그리고 CCR2 차단제 등을 사용하였을 뿐만 아니라 모든 경우에서 염증세포의 침윤이 감소되었기 때문에, MCP-1/CCR 시스템의 억제로 인한 효과가 염증세포의 침윤 감소로 인한 결과인지 아니면 각종 세포에서 MCP-1/CCR2 억제의 직접적인 결과인지를 감별할 수 없었다.

목적: 본 연구에서는 단핵구/대식세포의 영향이 배제된 상태에서 MCP-1/CCR2 시스템이 신섬유화에 미치는 직접적인 영향을 생체 내외 실험을 통하여 알아보고자 하였다.

방법: 생체 외 실험으로는 신세뇨관 세포를 대조군과 MCP-1 투여군 (10 ng/ml)으로 나누어 배양하였으며, CCR2 억제제인 RS102895 (2 및 10 μM) 또는 CCR2 siRNA (10, 25 및 50 nM)를 함께 처치한 실험도 하였다. 처치 72시간 후 각 군에서 세포를 수집하였다. 생체 내 실험으로는 두 가지 모델의 마우스를 사용하였다. 첫 번째 모델은 C57BL/6J 마우스로 36 마리를 6 그룹으로 나누었다: 그룹 1, 대조군 (PBS) (N = 6) (Control, Con); 그룹 2, liposome vehicle (LV) + lenti-empty virus 투여군 (N = 6); 그룹 3, liposome-clodronate (LC) + lenti-empty virus 투여군 (N = 6); 그룹 4, LV + lenti-MCP-1 virus 투여군 (N = 6); 그룹 5, LC + lenti-MCP-1 virus 투여군 (N = 6); 그리고 그룹 6, LC + lenti-MCP-1

virus + RS102895 투여군 (N = 6). LV와 LC는 200 μl PBS 용액에 혼합하여 5일 간격으로 4주간 꼬리정맥을 통하여 주사하였으며, lenti-empty와 lenti-MCP-1 virus (1.5 x 10^7 transfection units)는 5일 간격으로 3회 꼬리정맥으로 주사되었다. RS102895 (3 mg/kg/day)는 피하내로 삽입된 osmotic mini-pump를 이용하여 4주간 투여되었다. 두 번째 모델은 Cd11b-diphtheria toxin receptor (DTR) 마우스로, 이 형질전환 마우스에 DT를 투여하면 말초혈액 내 단핵구/대식세포가 제거된다. DT (N = 18) 또는 PBS (N = 6)를 3일 간격으로 복강 내로 투여하였으며, 첫 투여 1일과 3일 후 lenti-MCP-1 virus를 꼬리정맥으로 주입하였다. DT + lenti-MCP-1 virus를 투여한 마우스 중 6 마리에는 RS102895 (3 mg/kg/day)가 투여되었다. DTR 마우스는 첫 lenti-MCP-1 virus 투여 10일 후 희생하여 샘플을 채취하였다. 신세뇨관 세포와 신장 조직 내 fibronectin, type I collagen, 그리고 CCR2의 단백 발현은 Western blot을 이용하여 분석하였으며, fibronectin과 type I collagen의 mRNA 발현은 real-time PCR로 평가하였다. 혈청과 신장 조직 내 MCP-1 농도는 ELISA를 이용하여 측정하였으며, 말초혈액 내 단핵구 수를 확인하였다. 신섬유화 정도는 fibronectin과 type I collagen 항체를 이용한 면역 조직화학 염색과 Masson’s trichrome 염색으로 관찰하였다.

결과: MCP-1으로 자극한 신세뇨관 세포에서 fibronectin과 type I collagen의 단백 발현은 대조군에 비하여 의미있게 증가되었으며, 이러한 증가는 RS102895 또는 CCR2 siRNA에 의하여 의의있게 억제되었다. 대조군에 비하여 C57BL/6J 마우스에 LC를 투여하거나 DTR 마우스에 DT를 투여한 2일 후 말초혈액 내 단핵구가 유의하게 감소되었다. Lenti-MCP-1 virus 투여로 신장 조직 내 단핵구/대식세포의 침윤은 의미있게 증가되었으나, LC 또는 DT를 동시에 투여한 경우에는 신장 조직 내 단핵구/대식세포의 침윤이 의의있게 감소되었다. Lenti-MCP-1 virus 단독 투여군 뿐만 아니라 lenti-MCP-1 virus와 LC 또는 DT 동시에 투여한 마우스에서 fibronectin과 type I collagen의 단백 발현이 대조군에 비하여 유의하게 증가되었고, 이러한 증가는 CCR2 억제제인 RS102895에 의하여 의미있게 억제되었다. 면역 조직화학 염색 결과상 단핵구/대식세포의 침윤이 배제된 LC 또는 DT 투여 마우스에서도 fibronectin과 type I collagen의 발현이 의의있게 증가되었으며, 이러한 증가는 RS102895에 의하여 유의하게 억제되었다.

결론: 이상의 결과를 종합하여 볼 때, MCP-1/CCR2 시스템이 신세뇨관 세포에 직접적으로 작용하여 신섬유화를 유도할 것으로 생각되며,

MCP-1/CCR2 시스템의 억제가 MCP-1이 병태생리에 관여하는 것으로 알려져 있는 각종 신질환의 발생 및 진행 억제에 유용할 것으로 사료된다.

핵심되는 말: Monocyte chemoattractant protein-1, 신세뇨관 세포, 신섬유화, 세포외 기질, 단핵구/대식세포